

# Posttranslational Folding of Vesicular Stomatitis Virus G Protein in the ER: Involvement of Noncovalent and Covalent Complexes

Aravinda de Silva, Ineke Braakman, and Ari Helenius

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510-8002

**Abstract.** In this study, we show that posttranslational folding of Vesicular Stomatitis virus G protein subunits can involve noncovalent, multimeric complexes as transient intermediates. The complexes are heterogeneous in size (4–21S<sub>20,w</sub>), contain several G glycopolypeptides, and are associated with BiP/GRP78. The newly synthesized, partially intrachain disulfide-bonded G proteins enter these complexes immediately after chain termination, and are released 1–4 min later as fully oxidized, trimerization-competent monomers. These monomers are properly folded, judging by their binding of conformation-specific mAbs. When the G protein is translated in

the presence of DTT, it remains reduced, largely unfolded and aggregated in the ER, but it can fold successfully when the DTT is removed. In this case, contrary to normal folding, the aggregates become transiently disulfide cross-linked. We also demonstrated that the fidelity of the folding process is dependent on metabolic energy. Finally, we established that the G protein of the folding mutant of the Vesicular Stomatitis virus, ts045, is blocked at a relatively late step in the folding pathway and remains associated with oligomeric, BiP/GRP78-containing folding complexes.

**D**URING the folding process, newly synthesized proteins have an enormous number of conformational states as possible options. Instead of sampling all of these in a search for an energy minimum, folding occurs along defined pathways via specific conformational intermediates (Anfinsen and Scheraga, 1975; Creighton, 1986; Jaenicke, 1991). In living cells, the navigation of most proteins through the folding pathway depends critically on the action of molecular chaperones and folding enzymes (for reviews see Freedman, 1984; Pelham, 1986; Rothman, 1989; Gatenby and Ellis, 1990; Gething and Sambrook, 1992). These are thought to act by preventing nonproductive side reactions such as irreversible aggregation, and by accelerating slow steps in the folding process.

As a compartment specialized for protein folding and maturation, the lumen of the ER is particularly rich in resident chaperones and folding enzymes. Chaperones associate with folding intermediates and misfolded proteins, and are thought to facilitate protein translocation into the ER (Vogel et al., 1990), folding, oligomeric assembly, and sorting (for reviews see Rothman, 1989; Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). In the ER they include immunoglobulin heavy chain binding protein (BiP/GRP78)<sup>1</sup>

(an HSP70 analog) and GRP94 (an HSP90 analog). The folding enzymes include prolyl *cis-trans*-isomerases (Stamnes et al., 1991) and protein disulfide isomerase (PDI) (Freedman, 1984). PDI is a redox enzyme needed for the correct formation of disulfides (Freedman, 1984; Bulleid and Freedman, 1988). For many proteins synthesized in the ER, the sequential formation of disulfide bonds constitutes a requirement for normal folding, and for subsequent transport to the Golgi complex and beyond. To allow disulfide formation, the redox conditions inside the ER lumen are more oxidizing than in the cytosol.

To better understand protein folding in the ER, we have focused on viral membrane glycoproteins. The G protein of the Indiana strain of Vesicular Stomatitis virus (VSV) used in this study is, in its mature form, a noncovalently associated homotrimer with a subunit size of 67 kD (Kreis and Lodish, 1986; Doms et al., 1987). Each subunit contains a single membrane spanning domain, a short carboxy-terminal cytoplasmic tail and a large ectodomain with two N-linked oligosaccharides (Rothman and Lodish, 1977; Rose et al., 1980; Rose and Gallione, 1981). The ectodomain contains 12 cysteines which are thought to form intrachain disulfide bonds.

Pulse-chase studies have shown that G protein folding in the ER involves both co- and posttranslational events (Machamer et al., 1990; our unpublished results). After chain termination, incompletely disulfide-bonded folding intermediates are seen in association with BiP/GRP78. With a half-time of about 2 min the G proteins become fully oxi-

A. de Silva's present address is Department of Biology, University of Rochester, Rochester, NY 14627.

1. *Abbreviations used in this paper:* BiP, immunoglobulin heavy chain binding protein; HA, Influenza hemagglutinin; NEM, N-ethylmaleimide; VSV, Vesicular Stomatitis virus.

dized, lose their association with BiP/GRP78, and reach the native conformation, as judged by the binding of conformation-specific mAbs (Machamer et al., 1990). The formation of noncovalent, transport-competent homotrimers occurs with a half time of  $\sim 8$  min, and the trimers are selectively transported via the Golgi complex to the plasma membrane (Doms et al., 1987; Zagouras et al., 1991). If the G protein misfolds, as is the case for nonglycosylated G protein and some mutants, it enters aggregates that are either noncovalently associated or disulfide cross-linked (Gibson, 1979; Doms et al., 1988; Machamer and Rose, 1988a).

In this study, we have analyzed the folding of G protein in the ER of CHO15B cells. A pulse-chase approach was used in conjunction with sucrose gradient centrifugation, immunoprecipitation and SDS-PAGE of nonreduced samples. Our results show that folding of G protein subunits can involve transient, multimolecular complexes or aggregates, and that—depending on the conditions—these may be stabilized by aberrant interchain disulfide bonds. For VSV G protein the formation of such transient multimolecular complexes apparently is part of the normal folding process. Our results also demonstrate that the posttranslational folding of G protein, as previously seen for Influenza virus hemagglutinin (HA) (Braakman et al., 1992a, b), is an energy-requiring process, which can be easily manipulated by the addition or removal of DTT.

## Materials and Methods

### Cell Lines, Viruses, and Reagents

A mutant CHO cell line (CHO15B) was used because these cells lack the enzyme *N*-acetylglucosamine transferase I, so that the *N*-linked oligosaccharides do not get terminally glycosylated. This allows G proteins in the ER to be conveniently distinguished on SDS-PAGE from G protein that has reached the Golgi complex and beyond (Balch et al., 1986). Indiana serotype VSV wild-type virus was obtained from Dr. Jack Rose (Department of Pathology, Yale University, New Haven, CT). *N*-ethyl maleimide (NEM) was purchased from Sigma Chemical Co. (St. Louis, MO) and monensin from Calbiochem Corp. (La Jolla, California). mAb I-14 was obtained from Dr. Dough Lyles (Bowman Gray School of Medicine, Wake Forest, IL), the polyclonal anti-VSV serum from Dr. Jack Rose, and the anti binding protein (BiP) antibody from Dr. David Bole (Bole et al., 1986). The preparation of P5D4, the mAb against the tail of G protein has been described (Kreis and Lodish, 1986).

### Infection, Labeling, and Chase Conditions

CHO15B cells were grown and infected with VSV as previously described in detail (Balch et al., 1986). For complete details of labeling and chase conditions see (Braakman et al., 1991; 1992a, b). In brief: 90% confluent 60-mm dishes of CHO15B cells, infected with VSV at a multiplicity of infection (m.o.i.) of 20 at 37°C, were used 4–5 h after infection. Before labeling, cells were washed twice with PBS and then depleted for 15 min at 37°C in serum-free medium without methionine and cysteine. Each dish was pulsed with 50  $\mu$ Ci of [<sup>35</sup>S]methionine and 50  $\mu$ Ci of [<sup>35</sup>S]cysteine for the indicated time at 37°C. The pulse was terminated and the chase begun by washing once and then incubating at 37°C in serum-free medium containing 5 mM methionine, 5 mM cysteine, and 0.5 mM cycloheximide for the indicated times. Although cycloheximide was added during the chase, the amount of labeled G protein increased during the first 3 min. This phenomenon, previously discussed in detail (Braakman et al., 1991), is observed whenever the length of the radioactive pulse approaches the translation time of the analyzed polypeptide. The chase was terminated by washing the cells twice with ice-cold PBS containing 20 mM NEM. The cells were lysed by adding 1% Triton X-100 in MNT buffer (20 mM MES, 30 mM Tris, 100 mM NaCl, 1.25 mM EDTA, 1 mM EGTA), pH 7.4, containing 20 mM NEM. In experiments where BiP and associated proteins were immunoprecipitated, cells were lysed in 20 mM Tris, pH 7.5, 10 mM C<sub>12</sub>E<sub>8</sub>, 1 mM

MgCl<sub>2</sub>, and 20 mM NEM, containing 30 U/ml of apyrase (Sigma Chemical Co.) to deplete ATP levels in the lysate (Hurtley et al., 1989). To prevent proteolysis, the lysis buffers contained 1 mM PMSF and 10  $\mu$ g/ml each of chymostatin, leupeptin, antipain, and pepstatin. All lysates were spun for 5 min at 12,000 g to pellet nuclei and cell debris before further processing.

### Immunoprecipitations and SDS-PAGE

Immunoprecipitations were carried out as previously described (De Silva et al., 1990). Samples were prepared for SDS-PAGE and fluorographed as previously described (Balch et al., 1986).

### Velocity Gradient Centrifugation

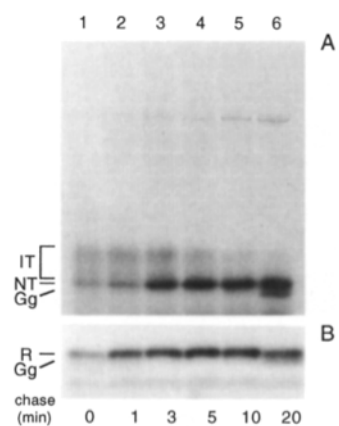
The centrifugation conditions used to determine the size of folding intermediates were as previously described (Doms et al., 1987) except that an SW40 rotor was used. In brief: the lysates were prepared in low pH (5.5) lysis buffer and layered over a 5–20% sucrose gradient (MNT, pH 5.8, and 0.5% TX100) with a 1 ml 60% sucrose cushion at the bottom of the tube. The gradient was centrifuged at 40,000 rpm for 17 h at 4°C. At the end of the run the gradients were fractionated from the bottom and the pH of the fractions was adjusted to 7. The distribution of G protein was followed by immunoprecipitation with a mixture of the polyclonal anti-VSV serum and the mAb P5D4. To check conformation, the different fractions were also immunoprecipitated with the mAb I-14.

## Results

### Conformational Maturation of G Protein

The folding of G protein in VSV (Indiana strain) infected CHO15B cells was analyzed using a pulse-chase approach that took advantage of the increase in electrophoretic mobility in oxidized G protein caused by the formation of intrachain disulfide cross-links (Machamer et al., 1990; Braakman et al., 1991). Infected CHO15B cells were pulsed with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 2 min and chased for different times up to 20 min. Before lysis and immunoprecipitation with anti-VSV antibodies, the cells were cooled and treated with NEM, which alkylates any remaining free sulfhydryl groups and thus prevents further disulfide bond formation (Creighton, 1986). The immunoprecipitates were subjected to SDS-PAGE under reducing and nonreducing conditions.

The G protein labeled during the 2-min pulse gave a heterogeneous banding pattern on the nonreducing gels (Fig. 1 A, lane 1). A diffuse set of bands, denoted IT for 'intermediates', corresponded to G proteins with an incomplete set of intramolecular disulfide bonds (Machamer et al., 1990). The faster migrating, sharper band (called NT for 'native') corresponded to the fully oxidized, untrimmed G protein. After 20 min of chase (Fig. 1 A, lane 6), a still faster migrating band (called Gg) appeared. This band represented the oxidized G proteins that had reached the Golgi complex and whose *N*-linked sugar moieties had been trimmed (Balch et al., 1986). No G protein was present at the top of the gel indicating that cross-linked aggregates of folding intermediates or misfolded forms of G protein did not arise. The higher molecular weight band that is present in lanes 3–6 is a background band unrelated to VSV G protein. When reduced before electrophoresis (Fig. 1 B), the same samples showed only one (Fig. 1 B, lanes 1–5) or two (Fig. 1 B, lane 6) G protein bands depending on whether any of the G protein had been trimmed in the Golgi complex. After 20 min of chase, about one third of G protein had reached the Golgi complex (Fig. 1 B, lane 6) judging by its faster mobility (Gg) (Balch et al., 1986).



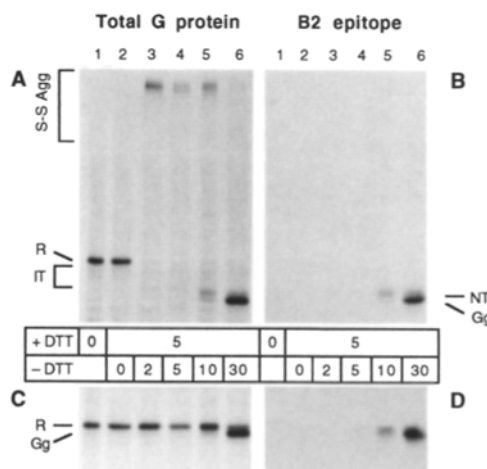
**Figure 1.** Time course of G protein oxidation. 5 h after infection with VSV, CHO15B cells were pulse labeled for 2 min at 37°C and chased for the indicated times. Detergent lysates from the cells were immunoprecipitated with a polyclonal antibody against VSV, and analyzed by 7.5% SDS-PAGE under nonreducing (A) and reducing (B) conditions. IT, folding intermediates; NT, native, completely oxidized G protein; R, completely reduced G protein; and Gg, G protein with trimmed carbohydrates, which has reached the Golgi. The higher molecular weight band that is present in lanes 3–6 is a background band unrelated to VSV G protein.

hydrates, which has reached the Golgi. The higher molecular weight band that is present in lanes 3–6 is a background band unrelated to VSV G protein.

Thus, in agreement with our previous findings in COS cells (Machamer et al., 1990), the G protein was converted to the fully oxidized form with a half time of ~1–3 min after the end of the pulse. That folding and disulfide bond formation already begin on the nascent chain, has been seen for immunoglobulin light chains (Bergman and Kuehl, 1979), for albumin (Peters and Davidson, 1982), and for HA (Braakman et al., 1991). No fully reduced, full-length G protein was ever seen after the pulse (Machamer et al., 1990) (Fig. 1 A, lane 1), suggesting that some of the disulfide bonds in G protein must already form before chain termination. Our previous studies have shown that the folding intermediates (IT) are transiently associated with BiP/GRP78 (Machamer et al., 1990), that the expression of conformational antigenic epitopes recognized by mAbs accompanies the oxidation and folding of G protein, and that the protein trimerizes before its arrival to the Golgi complex (Doms et al., 1987, 1988; Machamer et al., 1990). Taken together, these data provide the basic outline for the conformational maturation of G protein in the ER.

### DTT Inhibits the Folding and Transport of G Protein

We recently found that DTT, a membrane-permeable reducing agent, prevents co- and posttranslational disulfide bond formation in Influenza HA without markedly affecting its translation, translocation or covalent processing in the ER (Braakman et al., 1992a, b). To determine what consequences the addition of DTT would have on G protein folding and transport, we performed a pulse–chase experiment in the presence of 5 mM DTT. Fig. 2 A (lanes 1 and 2) shows that G protein was translated normally in the presence of DTT, but, judged by its slow migration on the gel identical to in vitro reduced G protein (Fig. 2 C, R, lanes 1 and 2), it failed to acquire disulfide bonds. That it remained incompletely folded was shown by the lack of precipitation with a mAb called I-14 (Fig. 2 B, lanes 1 and 2). I-14 recognizes epitope B-2, a conformational epitope expressed only in fully oxidized forms of G protein (Lefrancois and Lyles, 1982; Machamer et al., 1990). The N-linked oligosaccharide side chains of the reduced G protein remained untrimmed even after 40 min of chase in the presence of DTT, indicating that



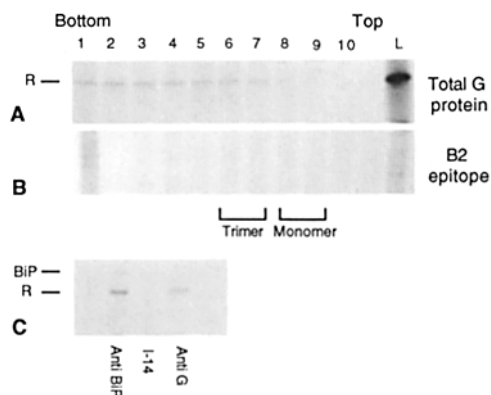
**Figure 2.** Effects of DTT on newly synthesized G protein. CHO15B cells infected with VSV were incubated with 5 mM DTT for 5 min before and during a 2-min pulse at 37°C. This was followed by chase periods with and without DTT, as indicated. Detergent lysates were immunoprecipitated with P5D4, a mAb against the tail of G protein (A and C) or I-14, a conformation-specific mAb against G protein (B and D), and analyzed by 7.5% SDS-PAGE under non-reducing (A and B) and reducing (C and D) conditions. S-S Agg, interchain disulfide-linked aggregates of G protein.

the protein was neither degraded nor transported to the Golgi (not shown). The formation of disulfide bonds was thus necessary for proper folding and for transport of G protein out of the ER.

### Covalent Complexes as Intermediates in Posttranslational Folding

When the DTT was removed, the reduced G protein that was trapped in the ER began to fold and was, after 10–30 min, transported to the Golgi complex in a fully oxidized and I-14 precipitable form (Fig. 2 A, lanes 5 and 6). Thus, DTT wash-out resulted in full recovery of correctly folded G protein. This demonstrated that while the G protein, like influenza HA (Braakman et al., 1992a), did require disulfide bond formation for folding, none of the disulfides had to form cotranslationally for G protein to acquire its normal conformation. As discussed above, the formation of disulfide bonds normally starts on the nascent chains with additional disulfides formed posttranslationally. Apparently proper folding could also be achieved with all disulfides forming on the full-length glycopolypeptide chain.

Although the final product of the posttranslational folding process after DTT removal was the correctly folded G protein, the pathway that led to it was not the same as that seen during normal folding. Immediately after the removal of DTT, large disulfide cross-linked complexes were formed, which did not enter the separating gel when the sample was not reduced (Fig. 2 A, lanes 3–5). These aggregates were, however, readily reduced to monomers when boiled with SDS and DTT, indicating that they were stabilized by interchain disulfide bonds (Fig. 2 C, lanes 3–5). About 10 min after DTT wash-out, G protein emerged from these covalent aggregates as untrimmed, oxidized monomers which had the gel mobility of normal folding intermediates and fully oxidized G protein (Fig. 2 A, lane 5). Clearly, the aberrant interchain disulfides were eliminated, and the aggregated G pro-



**Figure 3.** Reduced G protein occurs in large complexes bound to BiP. CHO15B cells infected with VSV were incubated with 5 mM DTT for 5 min before and during a 2-min pulse at 37°C. DTT was also included in the chase medium for 5 min. The detergent lysates were subjected to velocity centrifugation on sucrose gradients and the gradients were fractionated from the bottom. Lysate corresponding to 20% of the amount that was loaded on the gradient was also saved for precipitations (L). The fractions and the lysate sample (L) were precipitated with a mixture of P5D4 and a rabbit polyclonal antiserum (A) or with the conformation-specific mouse mAb I-14 (B). Samples were analyzed on 7.5% SDS-PAGE under non-reducing conditions. The position of the G protein monomer and G protein trimer are indicated. For C, the chase was for only 2 min in 5 mM DTT, after which the cells were lysed with the nonionic detergent C<sub>12</sub>E<sub>8</sub> in the presence of apyrase to deplete ATP and stabilize the interaction between BiP and associating forms of G protein. The lysate was then immunoprecipitated with a mAb against BiP, mAb I-14, and a polyclonal antibody against VSV.

teins thereby rescued (Fig. 2 A, Gg, lane 6). This provided a further illustration for the capacity of the ER machinery to reshuffle disulfide bonds and thus rescue misfolded proteins that had formed illegitimate covalent complexes (see Braakman et al., 1992b).

How can the formation of cross-linked aggregates after DTT removal be explained? Cross-linked aggregates do not normally occur as intermediates in the folding pathway. One possibility is that the reduced G protein subunits in the ER are already present as noncovalent aggregates. When the redox conditions return to normal and disulfide bonds begin to form, some interchain disulfides may arise simply because of the close proximity between the unfolded G protein polypeptides. To test this possibility, we subjected lysates from the DTT-treated cells to velocity centrifugation and analyzed the oligomeric state of the reduced G protein prior to DTT wash-out. Infected cells were labeled for 2 min and chased for 5 min in the continued presence of DTT. At the end of the chase, the cells were alkylated and solubilized in Triton X-100 and the postnuclear supernatant was analyzed on sucrose gradients containing Triton X-100. The G protein was recovered as heterogeneous particles with sedimentation coefficients ranging from 11S<sub>20,w</sub> to larger than 21S<sub>20,w</sub> (Fig. 3 A, fractions 1–6). Since monomeric and trimeric G proteins have sedimentation coefficients of 4S<sub>20,w</sub> (Fig. 3 A, fractions 8 and 9) and 8S<sub>20,w</sub> (fraction 7), respectively (Doms et al., 1987), it was apparent that the reduced G protein was present in heterogeneous, noncovalently associated complexes.

### BiP/GRP78 Is Associated with the G Protein Complexes

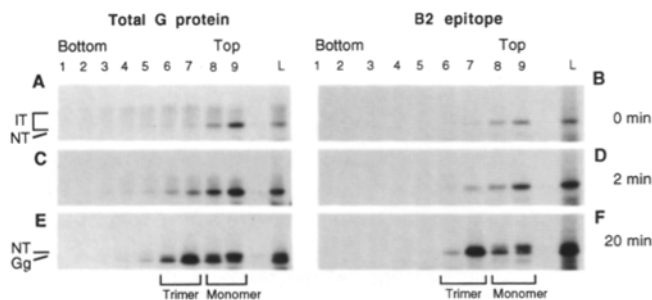
We have shown previously that BiP/GRP78 associates transiently and noncovalently with incompletely disulfide bonded folding intermediates of G protein (IT) (Machamer et al., 1990). To determine whether the DTT-reduced G protein complexes in the ER also bound BiP/GRP78, we prepared cell lysates as described above except that a milder lysis buffer was used and apyrase was included in the lysis buffer to hydrolyze the ATP (see Materials and Methods). ATP removal is necessary for preserving the bonds between G protein and BiP/GRP78 (Machamer et al., 1990). Immunoprecipitation was performed with a monoclonal anti-BiP antibody, with a polyclonal anti-VSV serum and with the I-14 conformation-specific mAb mentioned above. The results in Fig. 3 C show that the G protein complexes were efficiently precipitated with the anti-BiP antibody. They were also precipitated with the polyclonal anti-VSV serum, but not with the I-14 antibody. The DTT-reduced G protein was thus present in heterogeneous complexes up to several hundred kilodalton in molecular weight which associated with BiP/GRP78.

### Are Noncovalent Complexes Present during Normal Folding?

Although detectable amounts of covalent interchain disulfide cross-links do not occur during normal folding of G protein (Fig. 1 A), we were interested to determine whether some form of noncovalent assembly existed as transient intermediate. Cells infected with wild-type (wt) VSV were pulsed for 2 min and chased for 0, 2, or 20 min. At the end of each chase time, the cells were treated with NEM, lysed with Triton X-100, and the postnuclear supernatants were subjected to velocity gradient centrifugation. The G protein in the fractions was immunoprecipitated either with the polyclonal anti-VSV serum or with the mAb I-14, and analyzed on SDS-PAGE under nonreducing conditions.

After 0 and 2 min of chase (Fig. 4, A and C), the fully oxidized native form of the protein (NT) was recovered in fractions close to the top of the gradient in the position of monomeric G protein (Fig. 4, A and C, fractions 8 and 9; 4S<sub>20,w</sub>). This form of G protein was precipitated by the I-14 antibody (Fig. 4, B and D). In contrast, a large fraction of the incompletely disulfide-bonded folding intermediates (IT), which were not precipitated by I-14, were distributed across the gradient in heterogeneously sedimenting complexes (Fig. 4, A and C, fractions 2–9). Their sedimentation coefficients ranged from ~4 (fraction 9) to 21S<sub>20,w</sub> (fraction 2). Intermediates with different electrophoretic mobility—because of different degrees of oxidation—were randomly distributed among the heterogeneously sized complexes, suggesting that individual complexes contained G proteins in different stages of folding. Some of the G protein sedimented as monomers. These monomers appeared to be somewhat enriched in the faster migrating (i.e., more oxidized) forms of IT.

After 20 min of chase, most of the G protein was fully oxidized and recognized by the I-14 antibody (Fig. 4, E and F). It was recovered both as 4S monomers (Fig. 4, E and F, fractions 8 and 9) and as 8S trimers (fractions, 6, 7) (Doms et al., 1987). A large fraction of the trimers was trimmed, indicating that they were already transported out of the ER.



**Figure 4.** G protein folding intermediates occur in large complexes. CHO15B cells infected with VSV were pulsed and chased at 37°C for 0 (A and B), 2 (C and D), or 20 (E and F) min. Processing of the lysates was as in Fig. 3, A and B. Immunoprecipitation was with a mixture of P5D4 and a rabbit polyclonal antiserum. (A, C, and E) or with the conformation-specific mouse mAb I-14 (B, D, and F).

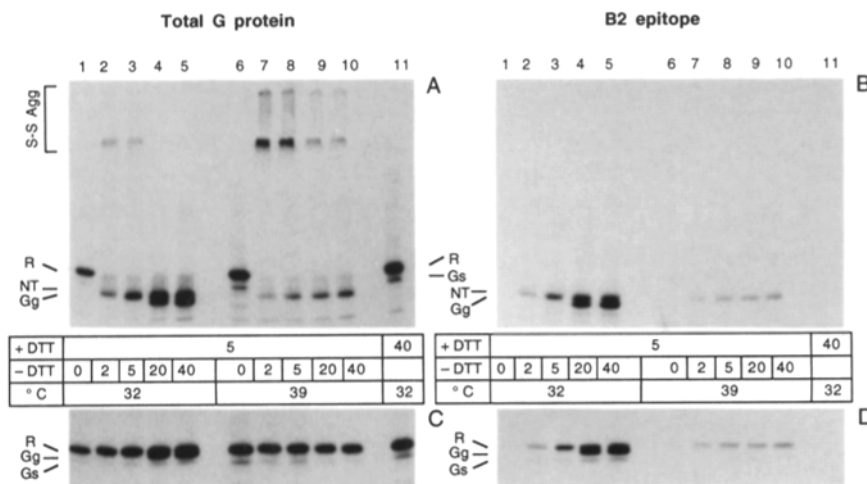
Taken together with our previous findings, these results indicated that immediately after release from the polysomes a large fraction of the full-length G protein subunits are associated with each other or with preformed complexes of heterogeneous sizes ranging from monomeric to ~700 kD. These G proteins then acquire more intrachain disulfide bonds until they are fully oxidized, whereupon they emerge from the complexes as native, folded monomers; they proceed to trimerize and to exit the ER. Since the G protein leaves the complexes several minutes before trimerization takes place, the complexes are not likely to play a role in oligomeric assembly but rather in the folding of the individual subunits. Nor is it likely that the complexes contain folding intermediates of other proteins than G protein. This is because VSV infection effectively shuts off host protein synthesis. It is, on the other hand, clear that the complexes contain some BiP/GRP78 because the time period during which the G protein associates with complexes is precisely the period during which G protein is known to interact with BiP/GRP78 (Machamer et al., 1990). Since ATP was not depleted, the BiP/GRP78 association was not preserved during the gradient analysis.

### Folding of ts045 G Protein

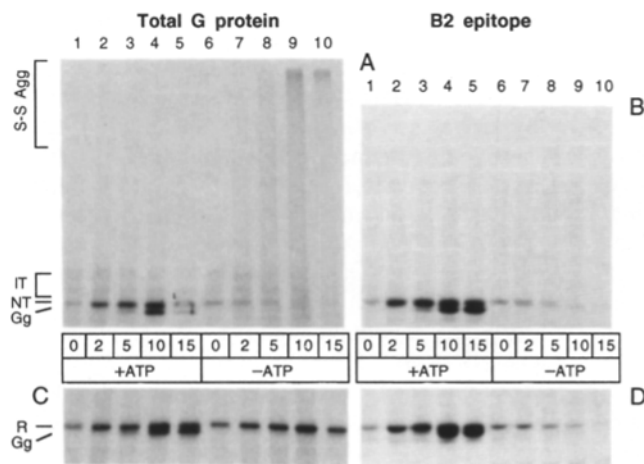
Having analyzed the main steps in the maturation of wt G protein, it was of interest to study the fate of the temperature-sensitive folding mutant ts045 which has been used extensively as a tool in membrane transport studies. The relevant mutation in the G protein is located in the ectodomain, a conversion of residue 204 from phenylalanine to serine (Gallione and Rose, 1985). At the permissive temperature (32°C), the G protein behaves like wt G protein. At nonpermissive temperature (39°C), the mutant protein forms non-covalent aggregates (Doms et al., 1987; De Silva et al., 1990; Machamer et al., 1990). In these, the B2 epitope is poorly expressed, one or more disulfide bonds are lacking, BiP/GRP78 is bound, and the protein remains trapped in the ER. Upon shifting to 32°C, the G protein completes its folding and assembles into transport-competent trimers (De Silva et al., 1990).

We first determined the effects of DTT at 32°C. Cells were infected with VSV ts045 virus, kept at 32°C, and pulse labeled in the presence of 5 mM DTT. As expected, the mutant G protein that was synthesized remained reduced and did not express the B2 epitope (Fig. 5, A and B, lane 1). Velocity centrifugation showed that a majority was present as noncovalent aggregates just like wt G protein synthesized in the presence of DTT. When DTT was removed from the medium at 32°C, the folding pathway was the same as that described above for wt G protein after DTT wash-out (Fig. 5, A and B, lanes 1–5).

When DTT was removed at 39°C (Fig. 5, A and B, lanes 7–10), initial folding events appeared to occur in the same way as at 32°C: disulfide cross-linked aggregates were formed (Fig. 5A, lane 7), and subsequently most of the inter-chain disulfides disappeared (lanes 8–10). Judging by the gel mobility of G protein, most or all of the normal intrachain disulfides were formed. However, the protein expressed only little of the B2 epitope and remained untrimmed (Fig. 5, A and B, lanes 7–10). Therefore, upon the removal of DTT at 39°C, the G protein fell just short of folding correctly in the ER. We showed previously that G protein remains trapped in BiP/GRP78 containing aggregates (Doms et al., 1987).



**Figure 5.** Effect of DTT on ts045 G protein. CHO15B cells infected with VSV ts045 were incubated with 5 mM DTT for 5 min before and during a 2-min pulse at 32°C (lane 1). The chase was done at 32°C (lanes 2–5) and 39°C (lanes 6–10) in medium with 5 mM DTT for the first 5 min and then in DTT-free medium for the indicated times at 32° and 39°C. Lane 11 is a sample of cells that were incubated with DTT in the chase medium for 40 min at 32°C to show that DTT blocks the transport of ts045 G protein. Processing was as in Fig. 2, except that a mixture of P5D4 and a rabbit polyclonal antiserum was used in A and C. Samples were subjected to 7.5% SDS-PAGE under nonreducing (A and B) and reducing (C and D) conditions. Gs refers to a minor byproduct of G protein that is formed due to a proteolytic cleavage near the membrane-spanning domain.



**Figure 6.** ATP dependence of folding of VSV G protein. CHO15B cells infected with VSV were pulse labeled for 1 min at 37°C and chased in medium with glucose (+ATP, lanes 1–5) or glucose-free medium containing 20 mM 2-deoxy-D-glucose and 10 mM sodium azide (–ATP, lanes 6–10) for the indicated times. Processing was as in Fig. 2. Analysis was by nonreducing (A and B) and reducing (C and D) 7.5% SDS-PAGE.

We concluded that the folding problem for ts045 G protein at 39°C is manifested at a relatively late state in the folding process, when the G protein is almost fully oxidized but still part of a complex. It remains trapped in a conformation similar to a late folding intermediate. This may explain why its phenotype is so easily reversed by temperature shifts.

### G Protein Folding Depends on Metabolic Energy

We showed previously that the thermo-reversion of ts045 G protein upon temperature shift-down is ATP dependent (Doms et al., 1987). More recently, we observed that the influenza HA can only fold correctly if there is access to metabolic energy, presumably in the form of ATP (Braakman et al., 1992b). To examine whether the folding of wt G protein is also ATP dependent, CHO15B cells were infected with VSV, and the newly synthesized proteins were pulse labeled with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine for 2 min. At the end of the pulse, glucose-deficient chase medium containing sodium azide and 2-deoxy-D-glucose was added to deplete the cells of ATP. Fig. 6 shows reduced and non-reduced samples derived from energy depleted and control cells.

We found that, upon energy depletion, the newly synthesized G protein rapidly coalesced into disulfide-linked aggregates that collected on top of the stacking gel (Fig. 6 A, lanes 6–10). Some monomeric IT as well as the NT form of G protein were transiently present during the early stages of ATP depletion, but they subsequently lost their conformation and became part of the aggregates. The new fully oxidized, B2-positive molecules present after short chase times disappeared concomitantly (Fig. 6, B and D, lanes 6–10). In control cells, the process of folding was normal (Fig. 6, lanes 1–5). These results showed that metabolic energy was required for the correct folding of newly synthesized G protein as well as for maintaining the conformational integrity of already folded and oxidized G protein present in the ER.

### Discussion

Multimolecular complexes and aggregates are commonly observed in the ER of living cells, but usually they contain only terminally misfolded proteins (Leavitt et al., 1977; Gibson, 1979; Doms et al., 1988; Machamer and Rose, 1988b; Hurtley et al., 1989; Tooze et al., 1989; Valetti et al., 1991). Under extreme conditions such aggregates can reach the size of inclusion bodies. While the underlying reasons for misfolding can be the presence of mutations in the proteins, the lack of N-linked glycosylation, or the incorporation of amino acid analogs, some proteins have a tendency to misfold spontaneously during the maturation process (Hurtley et al., 1989; Marquardt and Helenius, 1992). The aggregates that accumulate in the ER frequently contain interchain disulfide bonds and associate stably but noncovalently with BiP/GRP78 (Hurtley et al., 1989; Hurtley and Helenius, 1989). When more than one misfolded protein species is present, mixed aggregates are observed (Tooze et al., 1989; Marquardt and Helenius, 1992).

For the VSV G protein, aggregation was seen when glycosylation was inhibited by tunicamycin, and when mutations that modify the glycosylation sites had been introduced (Leavitt et al., 1977; Gibson, 1979; Gallione and Rose, 1985; Doms et al., 1988; Machamer and Rose, 1988b). With exception of the ts045 G protein, a natural mutant that can be reversed by a temperature shift (Flamand, 1970; Doms et al., 1987; De Silva et al., 1990), the aggregates have all been irreversible. The results presented here expand the repertoire of known conditions under which G protein complexes and aggregates occur in the ER, and demonstrate that G proteins undergoing normal folding may, in fact, transiently pass through an aggregated intermediate form.

We found that the majority of the G proteins was present in multimolecular complexes during the first 1–4-min period immediately after release from the polysomes, before reaching their fully oxidized, I-14 positive, monomeric conformation. They ranged in size from ~100 to 700 kD, and were, according to our previous results (Machamer et al., 1990), noncovalently associated with BiP/GRP78. Since this is the time period during which the proteins acquire many of their intrachain disulfide bonds, it is possible that productive folding may take place within the framework of these assemblies. Since some of the incompletely oxidized folding intermediates of G protein sedimented as monomers, we cannot, however, rule out the possibility that the G proteins in the complexes are in equilibrium with monomeric forms, and that the latter are the actively folding species. In this case, the progress of folding could depend on the transient release of G protein subunits from the aggregates. The failure of such protein recycling between an aggregated and a monomeric state could result in folding problems, as observed for instance after ATP depletion.

The heterogeneous size of the complexes argues against the notion that they represent homogeneous folding particles equivalent to those observed for homologs of HSP60 in mitochondria, bacteria, and chloroplasts (Cheng et al., 1989; Hubbard and Sander, 1991; Langer et al., 1992). As far as known, HSP60 has no homologs in the ER. The only chaperone that we know so far to be part of these ER complexes is BiP/GRP78 (Machamer et al., 1990).



It seems more likely that the complexes reflect the general tendency of folding intermediates to aggregate due to their hydrophobic surface properties. This phenomenon has been extensively analyzed during *in vitro* refolding studies with numerous proteins (Jaenicke, 1987; Mitraki and King, 1989; Kiefhaber et al., 1991). It is more pronounced at higher protein concentrations and at higher temperatures. While such aggregation *in vitro* generally competes with normal folding and lowers the refolding efficiency, there are cases where aggregates appear transiently during the refolding process as they do in our *in vivo* experiments (Brems, 1988). This suggests that even in the absence of chaperones, protein folding can proceed in spite of aggregate formation, if conditions are favorable.

While in the ER of the living cell the chaperones and folding enzymes help prevent aggregation and nonproductive interactions (see Rothman, 1989; Gatenby and Ellis, 1990; Hubbard and Sander, 1991; Gething and Sambrook, 1992), such reactions are not always entirely suppressed. Whether a given protein undergoes aggregation and misfolding or not, and whether the aggregates are permanent or transient, probably depends on many factors including the structure of the protein, the concentration and expression level, the rate of folding, and the concentration of available folding enzymes and chaperones. In cases such as the G protein, where aggregate formation is transient, the chaperones and folding enzymes may function to prevent the formation of irreversible intermolecular interactions within aggregates, and thus allow folding to proceed normally and efficiently.

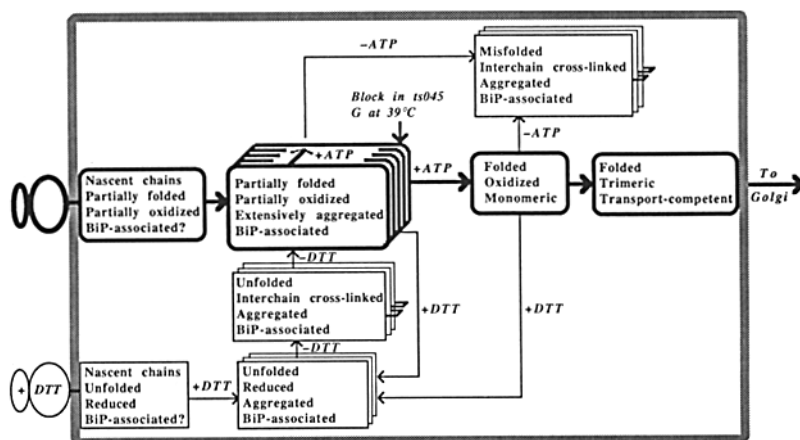
The view that aggregates represent problematic side products during protein folding in the ER is supported by our data on G protein folding in the presence of various inhibitors. We observed that disulfide cross-linked aggregates were formed when cells were depleted of ATP. Similar aggregates after ATP depletion were previously seen for influenza HA (Braakman et al., 1992b). Apparently, energy requiring processes such as the reversible association of BiP/GRP78 with the folding intermediates (Rothman, 1989) are needed to secure the fidelity of folding and to prevent the formation of covalent cross-links within aggregates. During protein folding in mitochondria and the cytosol, ATP is required to drive chaperones of the HSP60 and HSP70 families, both of which have a central role in protein folding (see Martin et al., 1991; Gething and Sambrook, 1992). We assume, although we have no direct evidence, that the involvement of

chaperones also explains the energy dependence of G protein folding in the ER.

How common are aggregated folding intermediates in living cells? In a recent paper on the maturation of thyroglobulin, Kim and Arvan (1991) reported that this large soluble glycoprotein passed through an aggregated intermediate while still in the lumen of the ER. Thyroglobulin is a large highly disulfide cross-linked protein which takes a long time to fold and mature within the ER. The hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3 has also recently been found to be present in some type of complex while still in the ER (Collins and Mottet, 1991). In this case it was suggested that the complexes were not only sites of subunit folding but also of tetrameric assembly. Studies with phage proteins that fold in the cytosol of bacteria have, moreover, implied a relationship between some aggregated states and folding intermediates (Mitraki and King, 1989; Mitraki et al., 1991). Transient aggregation of proteins during folding may thus be quite a common phenomenon, although we do not think, on the basis of preliminary experiments with HA, that it applies to all newly synthesized polypeptides in the ER.

While the demonstration of transient complexes during the normal folding process raises numerous questions about the mechanisms of folding, it also provides explanations for some puzzling observations. We noted some time ago that the fraction of misfolded HA generated as a side product during HA synthesis in CV-1 cells enters aggregates in less than a minute after chain-termination (Hurtley et al., 1989; Marquardt and Helenius, 1992). The HA stays aggregated thereafter as if selectively separated from the polypeptide chains that proceed to fold correctly. It was difficult to understand how they could be recognized as misfolded and segregated by the cellular system already before any of the HA molecules had had time to fold. Our present findings raise the possibility that all newly released chains may enter some type of complex after synthesis, but only those that are able to fold correctly can leave them. The misfolded molecules may be left behind in the folding complexes; when enough of them accumulate, they may coalesce to form inclusion bodies.

The formation of complexes between folding intermediates also helps to explain the origin of the transient interchain disulfides formed after DTT wash-out. The presence of DTT prevented disulfide bond formation and froze the folding pro-



**Figure 7.** G protein folding in the ER. This schematic representation of the various conformational and multimolecular forms that G protein can have in the ER is based on experiments reported here as well as in our previous reports (Doms et al., 1987, 1988; De Silva et al., 1990; Machamer et al., 1990). The normal folding pathway is indicated by bold boxes. The effects of DTT addition and removal and of ATP depletion are shown. Aggregates and complexes are shown as stacks and interchain disulfide bonds by brackets attached to the stacks. The step where the folding of ts045 G protein is blocked at 39°C is also indicated.

cess in an early stage when the G protein was in large BiP/GRP78 associated complexes. When the DTT was washed out, the redox conditions returned to normal, disulfide bond formation started, and aberrant interchain cross-links formed between neighboring molecules in the complexes. After a delay of ~10 min, the aberrant disulfides were eliminated in favor of correct intrachain disulfides and normal oxidized G protein subunits appeared. Reshuffling of disulfides within the complexes must have occurred to make this transition possible. The conformational rescue operation involved was most likely dependent on folding enzymes such as protein disulfide isomerase (Freedman, 1989; Noiva and Lennarz, 1992) and chaperones such as BiP/GRP78. These results provide an example of disulfide-exchange within the ER of living cells which leads to correct folding, and a demonstration that conformational modifications are possible even though proteins are aggregated.

Finally, the complex formation may have provided a partial explanation for the selectivity in the transport of G protein from the ER to the Golgi complex. While trimers of G protein can be transported efficiently to the Golgi, folding intermediates and misfolded proteins cannot (Rose and Doms, 1988; Hurlley and Helenius, 1989). We have recently observed (Hammond, C., and A. Helenius, unpublished results) that rapid transport of G protein from the ER to the intermediate compartment between the ER and the Golgi complex (Saraste and Kuismanen, 1984; Schweizer et al., 1988; Pelham, 1989) only occurs when the G protein is fully oxidized. The aggregated state of G protein during the folding phase may play a role in inhibiting its premature exit from the ER.

The stepwise progress of G protein folding in the ER is schematically depicted in Fig. 7. Normal folding (shown in bold boxes) begins on nascent chains, proceeds through a series of ATP dependent, posttranslational folding steps during which the G protein is present in aggregates associated with BiP/GRP78. The oxidized monomers that emerge are still dependent on ATP for their stability. They proceed to trimerize and exit from the ER. Fig. 7 also summarizes the effects of DTT addition and ATP removal, and indicates at which step in the pathway ts045 G protein folding is blocked at non-permissive temperature. When compared with influenza HA, whose folding in the ER is also quite well understood, G protein displays many similarities. The most important differences are the presence of transient aggregates and the transient binding of BiP/GRP78, neither of which we have been able to demonstrate for HA.

We thank J. Rose (Yale University, New Haven, CT), D. Lyles (Bowman Gray School of Medicine, Wake Forest, IL), T. Kreis (University of Geneva, Switzerland), and D. Bole (University of Michigan, Ann Arbor, MI) for generous gifts of reagents. J. Helenius is gratefully acknowledged for expert technical assistance, and H. Tan for photography. We thank members of the Mellman-Helenius laboratory for their advice and criticism.

This work was supported by grants GM38346 and NCI CA 46128 from the National Institutes of Health to A. Helenius. I. Braakman was supported by a post-doctoral fellowship from the Human Frontier Science Program Organization.

Received for publication 18 August 1992.

## References

Anfinsen, C. B., and H. A. Scheraga. 1975. Experimental and theoretical aspects of protein folding. *Adv. Protein Chem.* 29:205-300.

- Balch, W. E., M. M. Elliott, and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein between the endoplasmic reticulum and the Golgi. *J. Cell Biol.* 261:14681-14689.
- Bergman, L. W., and W. M. Kuehl. 1979. Formation of an intrachain disulfide bond on nascent immunoglobulin light chains. *J. Biol. Chem.* 254:8869-8876.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Braakman, I., H. Hoover-Litty, K. R. Wagner, and A. Helenius. 1991. Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* 114:401-411.
- Braakman, I., J. Helenius, and A. Helenius. 1992a. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1717-1722.
- Braakman, I., J. Helenius, and A. Helenius. 1992b. Role of ATP and disulfide bonds during protein folding in the endoplasmic reticulum. *Nature (Lond.)* 356:260-262.
- Brems, D. N. 1988. Solubility of different folding conformers of bovine growth hormone. *Biochemistry* 27:4541-4546.
- Bulleid, N. J., and R. B. Freedman. 1988. Defective co-translational formation of disulfide bonds in protein disulfide-isomerase-deficient microsomes. *Nature (Lond.)* 335:649-651.
- Cheng, M. Y., F.-U. Hartl, J. Martin, R. A. Pollock, F. Kalousek, W. Neupert, E. M. Hallberg, R. L. Hallberg, and A. L. Horwich. 1989. Mitochondrial heat-shock protein HSP60 is essential for assembly of proteins imported into yeast mitochondria. *Nature (Lond.)* 337:620-625.
- Collins, P. L., and G. Mottet. 1991. Homooligomerization of the hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3 occurs before the acquisition of correct intramolecular disulfide bonds and mature immunoreactivity. *J. Virol.* 65:2362-2371.
- Creighton, T. E. 1986. Disulfide bonds as probes of protein folding pathways. *Methods Enzymol.* 131:83-106.
- De Silva, A., W. E. Balch, and A. Helenius. 1990. Quality control in the endoplasmic reticulum: folding and misfolding of vesicular stomatitis virus G protein in cells and in vitro. *J. Cell Biol.* 111:857-866.
- Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105:1957-1969.
- Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. *J. Cell Biol.* 107:89-99.
- Ellis, R. J., and S. M. Van der Vies. 1991. Molecular chaperones. *Annu. Rev. Biochem.* 60:321-347.
- Flamand, A. 1970. Étude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensibles spontanés en groupes de complémentation. *J. Gen. Virol.* 8:187-195.
- Freedman, R. B. 1984. Native disulfide bond formation in protein biosynthesis: evidence for the role of protein disulfide isomerase. *Trends Biochem. Sci.* 9:438-441.
- Freedman, R. B. 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. *Cell* 57:1069-1072.
- Gallione, C. J., and J. K. Rose. 1985. A single amino acid substitution in a hydrophobic domain causes temperature-sensitive cell-surface transport of a mutant viral glycoprotein. *J. Virol.* 54:374-382.
- Gatenby, A. A., and R. J. Ellis. 1990. Chaperone function: the assembly of ribulose biphosphate carboxylase-oxygenase. *Annu. Rev. Cell Biol.* 6:125-149.
- Gething, M.-J., and J. Sambrook. 1992. Protein folding in the cell. *Nature (Lond.)* 355:33-45.
- Gibson, R., S. Schlesinger, and S. Kornfeld. 1979. The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* 254:3600-3607.
- Hubbard, T. J. P., and C. Sander. 1991. The role of heat-shock and chaperone proteins in protein folding: possible molecular mechanisms. *Protein Eng.* 4:711-717.
- Hurlley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277-307.
- Hurlley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland. 1989. Interactions of misfolded influenza hemagglutinin with binding protein (BiP). *J. Cell Biol.* 108:2117-2126.
- Jaenicke, R. 1987. Folding and association of proteins. *Prog. Biophys. Mol. Biol.* 49:117-237.
- Jaenicke, R. 1991. Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry* 30:3147-3161.
- Kieffhaber, T., R. Rudolph, H.-H. Kohler, and J. Buchner. 1991. Protein aggregation in vitro and in vivo: a quantitative model of the kinetic competition between folding and aggregation. *Bio/Technology* 9:825-829.
- Kim, P. S., and P. Arvan. 1991. Folding and assembly of newly synthesized thyroglobulin occurs in a pre-golgi compartment. *J. Biol. Chem.* 266:12412-12418.
- Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoproteins to the cell surface. *Cell* 46:929-937.



- Langer, T., C. Lu, H. Echols, J. Flanagan, M. K. Hayer, and F. U. Hartl. 1992. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature (Lond.)*. 356:683-689.
- Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Impaired intracellular migration and altered solubility of nonglycosylated glycoproteins of vesicular stomatitis virus and Sindbis virus. *J. Biol. Chem.* 252:9018-9023.
- Lefrançois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. 121:157-166.
- Machamer, C. E., and J. K. Rose. 1988a. Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J. Biol. Chem.* 263:5948-5954.
- Machamer, C. E., and J. K. Rose. 1988b. Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* 263:5955-5960.
- Machamer, C. E., R. W. Doms, D. G. Bole, A. Helenius, and J. K. Rose. 1990. Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. *J. Biol. Chem.* 265:6879-6883.
- Marquardt, T., and A. Helenius. 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.* 117:505-513.
- Martin, J., T. Langer, R. Boteva, A. Schramel, A. L. Horwich, and F.-U. Hartl. 1991. Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. *Nature (Lond.)*. 352:36-42.
- Mitraki, A., and J. King. 1989. Protein folding intermediates and inclusion body formation. *Bio/Technology*. 7:690-697.
- Mitraki, A., B. Fane, C. Haase-Pettingell, J. Sturtevant, and J. King. 1991. Global suppression of protein folding defects and inclusion body formation. *Science (Wash. DC.)*. 253:54-58.
- Noiva, R., and W. J. Lennarz. 1992. Protein disulfide isomerase. A multifunctional protein resident in the lumen of the endoplasmic reticulum. *J. Biol. Chem.* 267:3553-3556.
- Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell*. 46:959-961.
- Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1-23.
- Peters, T., Jr., and L. K. Davidson. 1982. The biosynthesis of rat serum albumin. *In vivo* studies on the formation of the disulfide bonds. *J. Biol. Chem.* 257:8847-8853.
- Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257-288.
- Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNA's encoding the Vesicular Stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* 39:519-528.
- Rose, J. K., W. J. Welch, B. M. Sefton, F. S. Esch, and N. C. Ling. 1980. Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. *Proc. Natl. Acad. Sci. USA*. 77:3884-3888.
- Rothman, J. E. 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell*. 59:591-601.
- Rothman, J. E., and H. F. Lodish. 1977. Synchronised transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (Lond.)*. 269:775-780.
- Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of semliki forest virus glycoproteins to the cell surface. *Cell*. 38:535-549.
- Schweizer, A., J. A. M. Fransen, T. Bächli, L. Ginsel, and H.-P. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the *cis*-side of the Golgi apparatus. *J. Cell Biol.* 107:1643-1653.
- Stamnes, M. A., B.-H. Shieh, L. Chuman, G. L. Harris, and C. S. Zuker. 1991. The cyclophilin homolog ninaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of Drosophila rhodopsins. *Cell*. 65:219-227.
- Tooze, J., H. F. Kern, S. D. Fuller, and K. E. Howell. 1989. Condensation-sorting events in the rough endoplasmic reticulum of exocrine pancreatic cells. *J. Cell Biol.* 109:35-50.
- Valetti, C., C. E. Brossi, C. Milstein, and R. Sitia. 1991. Russell bodies: a general response of secretory cells to synthesis of a mutant immunoglobulin which can neither exit from, nor be degraded in, the endoplasmic reticulum. *J. Cell Biol.* 4:983-994.
- Vogel, J. P., L. M. Misra, and M. D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* 110:1885-1895.
- Zagouras, P., A. Ruusala, and J. K. Rose. 1991. Dissociation and reassociation of oligomeric viral glycoprotein subunits in the endoplasmic reticulum. *J. Virol.* 65:1976-1984.